



**Universidade Nova de Lisboa**

**Instituto de Higiene e Medicina Tropical**

# New insights on nevirapine use: A mechanistic perspective of its toxic events

**Aline Teixeira Marinho**

**DISSERTAÇÃO PARA A OBTENÇÃO DO GRAU DE MESTRE EM CIÊNCIAS BIOMÉDICAS ESPECIALIDADE EM BIOLOGIA MOLECULAR EM MEDICINA TROPICAL E INTERNACIONAL**

**NOVEMBRO, 2013**

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# New insights on nevirapine use: A mechanistic perspective of its toxic events

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Dissertação apresentada para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ciências Biomédicas, especialização em Biologia Molecular em Medicina Tropical e Internacional.

Apoio financeiro da Fundação para a Ciência e a Tecnologia (PTDC/SAU-TOX/111663/2009 e PTDC/QUI-QUI/113910/2009).

**Bibliographic elements resulting from the dissertation:**

**Peer-reviewed Articles:**

**Marinho AT**, Rodrigues PM, Caixas U, Antunes AMM, Branco T, Harjivan SG, Marques MM, Monteiro EC, Pereira SA. Differences in nevirapine biotransformation as a factor for its sex-dependent dimorphic profile of adverse drug reactions. Journal of Antimicrobial Chemotherapy, 2013 (DOI:10.1093/jac/dkt359).

**Marinho AT**, Godinho ALA, Novais DA, Antunes AMM, Marques MM, Ramos T, Dias CG, Monteiro EC, Pereira SA. Development and validation of an HPLC-UV method for quantifying nevirapine and its main phase I metabolites in human blood. Analytical Methods (accepted with minor changes).

**Oral Communications in National Conferences:**

**Marinho AT**, Caixas U, Antunes AMM, Branco T, Faustino I, Marques MM, Monteiro EC, Pereira SA. Gender-related differences on Nevirapine biotransformation. XLIII Reunião Anual da Sociedade Portuguesa de Farmacologia/ XXXI Reunião de Farmacologia Clínica/ XII Reunião de Toxicologia, 2013, Porto.

**The participation in other ongoing projects of the research team originated the follow bibliographic elements:**

**Peer-reviewed Articles:**

Dias CG, Batuca JR, **Marinho AT**, Caixas U, Marques MM, Monteiro EC, Antunes AMM, Pereira SA. Quantification of the arylesterase activity of paraoxonase-1 in human blood. Anal Methods 2013 (DOI:10.1039/C3AY41527A).

Grilo NM, Antunes AM, Caixas U, **Marinho AT**, Charneira C, Conceição Oliveira M, Monteiro EC, Matilde Marques M, Pereira SA. Monitoring abacavir bioactivation in humans: Screening for an aldehyde metabolite. Toxicol Lett 2013, 219:59-64.

**Awards:**

Dias CG, **Marinho AT**, Antunes AMM, Caixas U, Branco T, Marques MM, Monteiro EC, Batuca JR, Pereira SA. Best Oral Presentation on "2nd International Conference on Occupational & Environmental Toxicology". Journals of Toxicology and Environmental Health and Taylor & Francis Group.

Rodrigues PM, Conde SV, Sacramento JF, **Marinho AT**, Ribeiro MJ, Antunes AMM, Marques MM, Monteiro EC, Pereira SA. Honorable mention for best poster on the "5.0 iMed Conference", about "New insights into personalized medicine – drug response variation in type II diabetes. The nevirapine story."

**Proceedings of International Conferences:**

Miranda JP, Pinheiro PF, **Marinho AT**, Harjivan SG, Castro M, Pereira SA, Antunes AMM, Marques MM. 3D hepatocyte cultures: A useful tool in the study of Nevirapine bioactivation and toxicity. Toxicology Letters 2013, 221, S174.

**Oral Communications in International Conferences:**

Miranda JP, Pinheiro PF, **Marinho AT**, Harjivan SG, Castro M, Pereira SA, Antunes AMM, Marques MM. 3D hepatocyte cultures: A useful tool in the study of Nevirapine bioactivation and toxicity. Eurotox, 2013, Switzerland.

Dias CG, **Marinho AT**, Antunes AMM, Caixas U, Branco T, Marques MM, Monteiro EC, Batuca JR, Pereira SA. Effect of chronic exposure to the antiretroviral drug nevirapine on Paraoxonase-1 activities in HIV-infected patients. 2nd International Conference on Occupational & Environmental Toxicology, 2013, Oporto.

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**Oral Communications on National Conferences:**

Rodrigues PM, Conde SV, **Marinho AT**, Antunes AMM, Marques MM, Monteiro EC, Pereira SA. Nevirapine biotransformation on type 2 diabetes. XLIII Reunião Anual da Sociedade Portuguesa de Farmacologia/ XXXI Reunião de Farmacologia Clínica/ XII Reunião de Toxicologia, 2013, Porto.

Grilo NM, Antunes AMM, Caixas U, Charneira C, **Marinho AT**, Oliveira MC, Marques MM, Monteiro EC, Pereira SA. An aldehyde metabolite as a possible factor on abacavir cardiotoxicity. XLIII Reunião Anual da Sociedade Portuguesa de Farmacologia/ XXXI Reunião de Farmacologia Clínica/ XII Reunião de Toxicologia, 2013, Porto.

**Poster in National Conferences:**

Rodrigues PM, Conde SV, Sacramento JF, **Marinho AT**, Ribeiro MJ, Antunes AMM, Marques MM, Monteiro EC, Pereira SA. New insights on personalized medicine – drug response variation in type II diabetes. The Nevirapine Story. Fundação AstraZeneca Innovate Competition – iMed, 2013, Lisboa.

*Dedicatória*

**Ao amor da minha vida,**
Ricardo Cerqueira

**Ao meu pai,**
Luíz Eduardo Marinho

**À memória da minha mãe,**
Lúcia Marinho

**Às minhas queridas Avós,**
Armandina e Nadege

**Aos meus tios,**
Marília e Márcio,

**Aos meus primos,**
Vanessa, João Dias e Deborah

**Às amigas,**
Rita Beijinho e Débora Matos

**Ao meu Puca.**

# AGRADECIMENTOS

Em primeiro lugar, um agradecimento especial à Professora Doutora Sofia Pereira, minha orientadora, sem a qual todo este mestrado não seria possível. Sendo difícil expressar toda a minha gratidão em algumas linhas, agradeço-lhe sinceramente por tudo o que aprendi ao longo destes quase 3 anos, pela sua paciência com quem sabe tão pouco, por acreditar em mim, por compreender as minhas limitações e pela sua amizade. É um enorme privilégio poder trabalhar e aprender com alguém assim.

Gostaria também de agradecer à Professora Doutora Maria Emília Monteiro, responsável pelo Departamento de Farmacologia, por estar sempre disponível para ajudar e pelo seu exemplo de liderança e dedicação.

Gostaria de agradecer também à Professora Doutora Alexandra Antunes e à Professora Doutora Matilde Marques, que sempre me receberem tão bem no Laboratório de Química. Muito obrigado por tudo o que me ensinaram, pelo vosso incansável empenho e dedicação e por tornarem possível uma parte fundamental deste trabalho.

Às médicas envolvidas neste trabalho, Dra. Umbelina Caixas e Dra. Teresa Branco, muito obrigado pela vossa simpatia, pela disponibilidade e pelo contributo essencial que deram a este trabalho, que também é vosso.

Os meus sinceros agradecimentos também a todas as pessoas que trabalham comigo no Laboratório de Farmacologia, em especial às minhas amigas "tóxicas", Nádia Grilo, Patrícia Rodrigues e Clara Dias. Agradeço também à Maria João Ribeiro e à Joana Sacramento, à Inês Faustino, à Doutora Joana Batuca, à Professora Sílvia Conde, à Doutora Judit Morello, à Doutora Lucília Diogo, à Doutora Rita Nunes, à Doutora Teresa Monteiro e à Teresa Lopes pelo bom ambiente que sempre criaram no nosso laboratório. Muito obrigada a todas pela vossa amizade e apoio e por criarem no nosso departamento um ambiente único e tão especial.

Por fim queria agradecer à minha família, em especial ao meu pai e às minhas avós. Muito obrigado pelo vosso amor incondicional, pelo vosso carinho, paciência e compreensão. Agradeço ainda ao Ricardo, por todo o amor que me dedica, nem tenho palavras para descrever o quanto me sinto abençoada por poder partilhar os meus dias com alguém tão especial.

## RESUMO

## NOVOS DESENVOLVIMENTOS NO USO DA NEVIRAPINA: UMA PERSPECTIVA MECANICISTA DOS SEUS EFEITOS TÓXICOS

## AUTOR: ALINE TEIXEIRA MARINHO

**Introdução:** A nevirapina (NVP) é um fármaco amplamente utilizado para o tratamento da infecção pelo vírus da imunodeficiência humana de tipo 1 (VIH-1), no entanto, a sua utilização na terapêutica crónica tem sido associada à toxicidade hepática e cutânea. O sexo feminino é um factor de risco para o desenvolvimento destes eventos tóxicos, mas as razões para essa diferença entre o sexo feminino e masculino não estão completamente esclarecidas. Diferenças na biotransformação da NVP e na formação de metabolitos tóxicos podem ser as causas subjacentes. O presente trabalho teve como objectivo explorar as diferenças entre homens e mulheres na biotransformação da NVP, como um potencial factor de toxicidade induzida por este fármaco anti-retroviral.

**Materiais e Métodos:** Todos os indivíduos incluídos no presente estudo eram adultos com infecção por VIH-1 confirmada, tratados com 400 mg de NVP uma vez ao dia, durante pelo menos 1 mês. Foram colhidas amostras de sangue e os níveis de NVP e dos metabolitos de fase I foram determinados por cromatografia líquida de alta performance. Os dados antropométricos e clínicos e os perfis de metabolitos foram avaliados de forma a averiguar possíveis diferenças relacionadas com o sexo dos indivíduos.

**Resultados:** Foram incluídos 52 doentes (63% do sexo masculino). O peso corporal foi inferior nas mulheres ($p = 0.028$) e o sexo feminino foi associado a maiores níveis de fosfatase alcalina ($p = 0.036$) e lactato desidrogenase ($p = 0.037$). Os níveis plasmáticos de NVP ($p = 0.030$) e 3-hidroxi-NVP ($p = 0.035$), assim como as proporções de 12-hidroxi-NVP ($p = 0.037$) e 3-hidroxi-NVP ($p = 0.001$) foram maiores nas mulheres, quando ajustados pelo peso corporal dos indivíduos.

**Discussão:** Existem diferenças na biotransformação da NVP entre homens e mulheres, particularmente na formação de 12-hidroxi-NVP e 3-hidroxi-NVP. Estes resultados apontam para uma formação de metabolitos reactivos, que é dependente do sexo e que pode contribuir para o perfil de dimorfismo sexual associado às reacções tóxicas induzidas pela NVP.



PALAVRAS-CHAVE: nevirapina, toxicologia, diferenças associadas ao género, farmacocinética, biotransformação de fase I.

## ABSTRACT

## NEW INSIGHTS ON NEVIRAPINE USE: A MECHANISTIC PERSPECTIVE OF ITS TOXIC EVENTS

## AUTHOR: ALINE TEIXEIRA MARINHO

**Introduction:** Nevirapine (NVP) is widely used for the treatment of human immunodeficiency virus type 1 (HIV-1) infection; however, its chronic use has been associated with severe liver and skin toxicity. Women are at increased risk for these toxic events, but the reasons for the sex-related differences are unclear. Disparities in the biotransformation of NVP and the generation of toxic metabolites between men and women might be the underlying cause. The present work aimed to explore sex differences in NVP biotransformation as a potential factor in NVP-induced toxicity.

**Materials and Methods:** All included subjects were HIV-1-infected adults who had been receiving 400 mg of NVP once daily for at least 1 month. Blood samples were collected and the levels of NVP and its phase I metabolites were quantified by high-performance liquid chromatography. Anthropometric and clinical data, and NVP metabolite profiles, were assessed for sex-related differences.

**Results:** A total of 52 patients were included (63% were men). Body weight was lower in women ($p = 0.028$) and female sex was associated with higher alkaline phosphatase ($p = 0.036$) and lactate dehydrogenase ($p = 0.037$) levels. The plasma concentrations of NVP ($p = 0.030$) and the metabolite 3-hydroxy-NVP ($p = 0.035$), as well as the proportions of the metabolites 12-hydroxy-NVP ($p = 0.037$) and 3-hydroxy-NVP ($p = 0.001$), were higher in women, when adjusted for body weight.

**Conclusions:** There was a sex-dependent variation in NVP biotransformation, particularly in the generation of the 12-hydroxy-NVP and 3-hydroxy-NVP metabolites. These data are consistent with the sex-dependent formation of toxic reactive metabolites, which may contribute to the sex-dependent dimorphic profile of NVP toxicity.


KEYWORDS: nevirapine, toxicology, sex-differences, pharmacokinetics, phase I biotransformation.

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## ABBREVIATIONS

ALKP – alkaline phosphatase

ALT – alanine amino-transferase

APS – adenosine 5'-phosphosulfate

cART – combined antiretroviral therapy

CCHCR1 – coiled-coil alpha-helical rod protein 1 gene

CV – coefficient of variation

CYP – cytochrome P450

FDA – Food and Drug Administration

GGT – gamma-glutamyltransferase

GSH – glutathione

HIV – human immunodeficiency virus

HLA – human leukocyte antigen

HPLC – high-performance liquid chromatography

LDH – lactate dehydrogenase

LLOQ – lower limit of quantitation

MHC – major histocompatibility complex

NNRTI – non nucleoside reverse transcriptase inhibitor

NVP – nevirapine

PAPS – 3'-phosphoadenosine-5'-phosphosulfate

PPARs – peroxisome proliferator-activated receptors

QC – quality control

SEM – standard error of the mean

SNP – single nucleotide polymorphisms

SULTs – sulfotransferases

WHO – World Health Organization

2-OH-NVP – 2-hydroxy-nevirapine

3-OH-NVP – 3-hydroxy-nevirapine

4-COOH-NVP – 4-carboxy-nevirapine

8-OH-NVP – 8-hydroxy-nevirapine

12-OH-NVP – 12-hydroxy-nevirapine

# 1. Introduction

## 1.1. The human immunodeficiency virus infection and the combined antiretroviral therapy

### 1.1.1. The burden of the human immunodeficiency virus epidemics and natural history of the infection

The human immunodeficiency virus (HIV) infection is a significant public health issue at a global scale (Arts and Hazuda, 2012, Piot and Quinn, 2013). This infection is an epidemic in several developed and developing regions worldwide and a leading cause of death in Africa (Ortblad et al., 2013, Piot and Quinn, 2013). For instance, the prevalence of this infection is as high as 31% of HIV-infected adults in Swaziland and 25% in Botswana (Piot and Quinn, 2013). Also, in Mozambique and in South Africa 11% and 17% of adults are HIV-infected. Moreover, the access to combined antiretroviral therapy (cART) is quite heterogeneous across countries. In Mozambique the cART coverage do not reach 50% of patients with advanced infection, in South Africa this coverage is slightly higher (66%). In Portugal this scenario is completely different: the prevalence of HIV infection is 0.7%, nonetheless one of the highest percentages among European countries, the access to treatment is guaranteed by the Portuguese *National Health Service* (WHO, 2011 ). Indeed, the access to cART changed dramatically the prognosis of this infection, which in nowadays is perceived as a chronic disease, particularly in developed countries (Mehellou and De Clercq, 2010).

The natural history of HIV-1 infection is composed by three main phases: the acute or primary infection, the asymptomatic stage and the symptomatic stage (Touloumi and Hatzakis, 2000). During the primary infection, the individual may present clinical symptoms as fever or lymphadenopathy. This first stage is also characterized by high levels of plasma viral load and a significant decrease in $CD4^+$ T lymphocyte counts. These $CD4^+$ T lymphocytes will return to a near normal count and the viral load will decline and stabilizes after a few months of infection. Subsequently, the asymptomatic period is characterized by a slow reduction of $CD4^+$ counts; this phase can persist for a decade depending on each individual's health status (Touloumi and Hatzakis, 2000). Afterwards, the number of immune cells drops significantly and the individuals initiate the symptomatic phase. As long as $CD4^+$ counts drop below 200 cells/µL, most of AIDS-

defining conditions arise. The progression to AIDS is associated with several opportunistic infections, HIV-related encephalopathy and AIDS-defining malignancies (Touloumi and Hatzakis, 2000, Schneider et al., 2008).

### 1.1.2. The antiretroviral drugs

The *World Health Organization* (WHO), on its publication *antiretroviral therapy for HIV infection in adults and adolescents – recommendations for a public health approach*, advocates the initiation of cART for adults with $CD4^+$ counts below 350 cells/µL. This recommendation is also applicable to pregnant women and adolescents, irrespectively to the absence of symptoms. This recommendation extends to those with advanced clinical disease, regardless their $CD4^+$ cells counts. Also, patients with co-morbidities, such as tuberculosis or hepatitis B, should initiate cART if there is active tuberculosis disease or if they require treatment for hepatitis B infection, independently of their $CD4^+$ counts (WHO, 2010).

Antiretroviral drugs are distributed by several classes (Table 1) according to their mechanism of action (Figure 1). These drugs are prescribed in combination of three, two of which are nucleoside reverse transcriptase inhibitors or tenofovir, plus one of the following options: a non-nucleoside reverse transcriptase inhibitor (NNRTI), one protease inhibitor boosted with ritonavir, or one integrase inhibitor (WHO, 2010, Guerreiro et al., 2012). Nonetheless, these combinations might need to be altered, mainly due to virological failure or drug-induced toxicity, in these situations drugs belonging to other classes might be indicated such as fusion inhibitors or CCR5 co-receptor antagonists (WHO, 2010, Arts and Hazuda, 2012, Guerreiro et al., 2012, Hartman and Buckheit, 2012).

In the last years, more than thirty anti-HIV drugs were introduced in the market. However, the search for new molecules with antiretroviral activity and new therapeutic targets and mechanisms of action is still ongoing (Hartman and Buckheit, 2012, Li et al., 2013). For instance, the use of ibalizumab, a monoclonal antibody that binds to CD4 receptor has been showing promising results on viral replication control (Bruno and Jacobson, 2010), inclusively being investigated the possibility of ibalizumab use for pre-exposition prophylaxis (Abraham and Gulick, 2012).

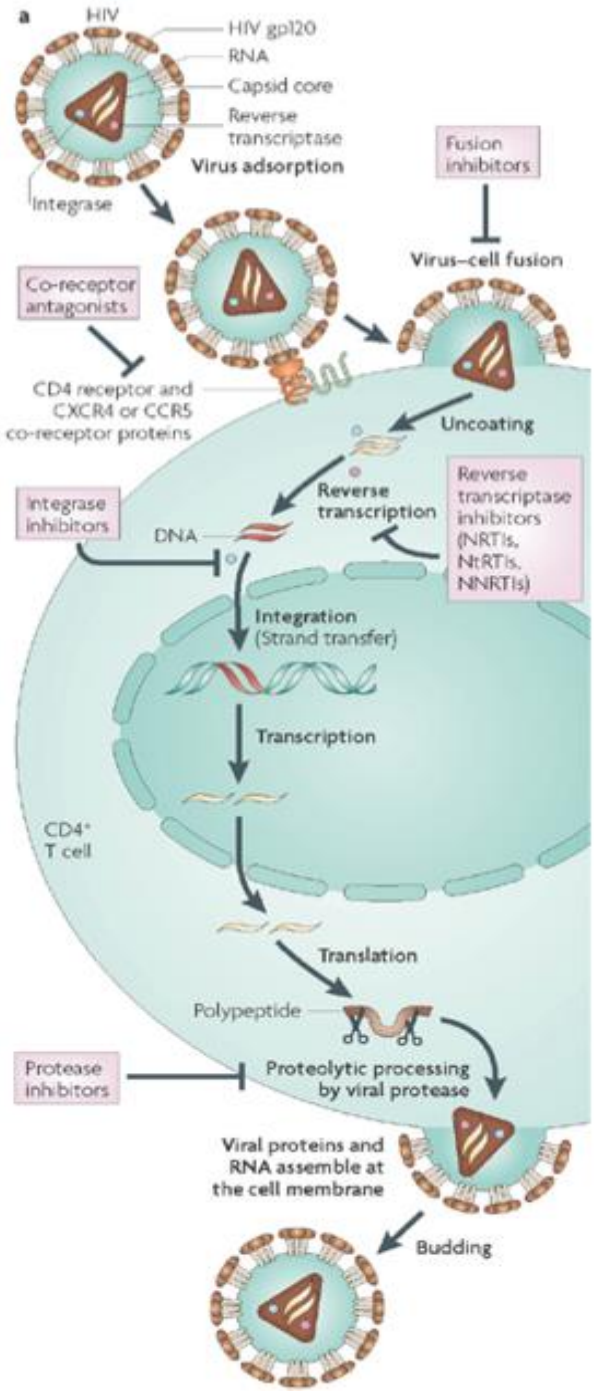



**Figure 1 Schematic overview of the human immunodefiency virus (HIV) replication cycle and therapeutic targets of antiretrovirals.** Mechanism of action for each class of antiretroviral drugs throughout the different stages of HIV replication cycle. In:De Clercq, 2007.

**Table 1 Antiretroviral drugs classes and their mechanism of action**

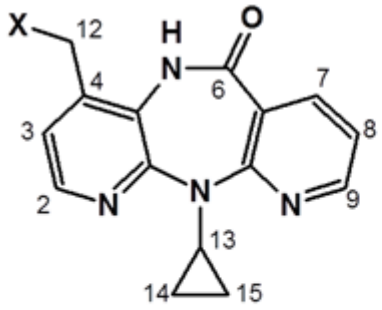
| | Antiretroviral drugs classes | Mechanism of action | Examples | References |
|---|---|---|---|---|
| Reverse transcriptase inhibitors | Nucleoside analogues | These analogues of deoxynucleosides need to be phosphorylated by cellular kinases to be pharmacologically active. These compounds are incorporated on growing pro-viral DNA chain, however due to the absence of 3'-hydroxyl group on its deoxyribose moiety these drugs inhibit the formation of 3'-5'-phosphodiester bound, leading to chain termination. | Abacavir, lamivudine, Didanosine, Stavudine, Emtricitabine | Hart et al., 1992; Richman, 2001 |
| | Nucleotide analogue | Tenofovir has the same mechanism of action as nucleoside analogues but only require two phosphorylation steps. | Tenofovir | Arts and Hazuda, 2012 |
| | Non-nucleos(t)ide analogues | These drugs bind to HIV-1 reverse transcriptase, in a non-competitive manner, near to active site, leading to inhibition of its activity. | *Nevirapine*, Efavirenz | De Clercq, 2004; Arts and Hazuda, 2012 |
| Protease inhibitors | | Protease inhibitors prevent virion maturation through the inhibition of the enzyme responsible for the cleavage of HIV gag and gag-pol polyproteins, which are the precursors of structural and non-structural HIV proteins. | Lopinavir, Saquinavir, Indinavir | Arts and Hazuda, 2012 |
| Integrase inhibitors | | These drugs bind to the integrase-viral DNA complex and sequester magnesium in the integrase active site, thus preventing the integration of pro-viral DNA into cell genome. | Raltegravir Dolutegravir | Grobler et al., 2002, Hartman and Buckheit, 2012, Flexner and Saag, 2013 |
| Fusion inhibitors | | Fusion inhibitors bind to the viral protein gp41 and prevent the HIV fusion with cell membrane. | Enfuvirtide | Arts and Hazuda, 2012, Hartman and Buckheit, 2012 |
| CCR5 co-receptor antagonists | | Maraviroc bind to CCR5 in an allosteric site, inducing conformational changes that avoid CCR5 interaction with HIV-1 gp120, and consequently inhibit viral entry through this co-receptor. | Maraviroc | Arts and Hazuda, 2012, Hartman and Buckheit, 2012 |

Finally, the availability of cART has changed the prognosis of HIV infection in properly medicated patients, from a lethal disease into a chronic condition. However, HIV-positive individuals still face obstacles associated with chronic cART use, with particular concern for long-term adverse events (Powles et al., 2009).

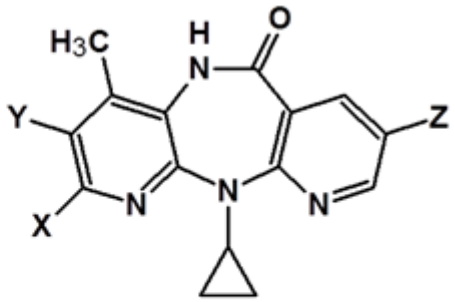
## 1.2. A focus on nevirapine: pharmacology, toxicology and pharmacogenetics

### 1.2.1. Pharmacology of nevirapine

Nevirapine (NVP, Figure 2) was the first NNRTI, approved by the American *Food and Drug Administration* (FDA) in 1996 for the treatment of HIV-1 infection as part of cART (Bowersox, 1996). Currently, NVP remains the most prescribed NNRTI worldwide and the most prescribed antiretroviral drug in countries with limited economic resources, mainly due to its efficacy, low cost and availability as a generic prescription (Ades et al., 2000, Lockman et al., 2007). In Portugal, NVP is considered a first-line drug for the treatment of HIV-1 infection in adults and adolescents, according to national guidelines for treatment of HIV infection (Guerreiro et al., 2012).




1. X=H; Nevirapine (NVP)
5. X=OH; 12-Hydroxy-NVP




2. X=OH, Y=Z=H; 2-Hydroxy-NVP
3. X=Z=H, Y=OH; 3-Hydroxy-NVP
4. X=Y=H, Z=OH; 8-Hydroxy-NVP

**Figure 2 Structures of nevirapine and its phase I metabolites.**

However, one of the most significant benefits of NVP is its efficacy in the prevention of vertical transmission of HIV-1 infection, with the drug being commonly prescribed to pregnant women and their children (Guay et al., 1999, Ades et al., 2000, Taha et al., 2004, Medrano et al., 2008, Guerreiro et al., 2012). Administration of oral single dose of NVP to HIV-infected pregnant women at the onset of labor and to their babies within 3 days of birth significantly reduce the risk of mother-to-child transmission of HIV (Guay et al., 1999, Lallemant et al., 2004).

Additionally, the favorable metabolic profile is another important advantage of NVP (Ruiz et al., 2001, Clotet et al., 2003, van Leeuwen et al., 2003, van Leth et al., 2004 , Batuca et al., 2012), rendering it suitable for use in individuals with dyslipidemia, diabetes or metabolic syndrome. Indeed, NVP has been associated with a better lipid profile in naive (Clotet et al., 2003, van Leth et al., 2004 ) and experienced patients (Ruiz et al., 2001, Clotet et al., 2003) as well as in pregnant women (Floridia et al., 2009) and in uninfected newborns (Sankatsing et al., 2007).

Furthermore, the low incidence of adverse events in the central nervous system allows NVP use in the context of narcotic drugs abuse or neuropsychiatric disorders (Medrano et al., 2008), by opposition of the other first line NNRTI, efavirenz, which is not recommended in these situations due to its high incidence of adverse events on the central nervous system (Núñez et al., 2002).

Moreover, the availability of a new extended-release formulation of NVP, allowing more convenient once-daily dosing, might improve adherence to treatment and avoid virological failure (Ward and Slim, 2013). All these advantages and benefits have guaranteed to NVP-based cART a relevant role in HIV-1 treatment strategies.

Regarding its pharmacokinetic properties, NVP is a highly hydrophobic molecule and is rapidly absorbed after oral administration, being its bioavailability greater than 90%. NVP is approximately 60% bound to plasma proteins, easily crosses the blood-brain barrier and placenta and is excreted in breast milk (Mirochnick et al., 2000, Elias and Nelson, 2013).

This drug is extensively biotransformed by cytochrome P450 (CYP) into five phase I metabolites: 2-hydroxy-nevirapine (2-OH-NVP), 3-hydroxy-nevirapine (3-OH-NVP), 8-hydroxy-nevirapine (8-OH-NVP), 12-hydroxy-nevirapine (12-OH-NVP) and 4-carboxy-nevirapine (4-COOH-NVP) ((Erickson et al., 1999, Riska et al., 1999a, Riska et al.,

1999b); Figures 2 and 3). These hydroxylated NVP metabolites undergo subsequent phase II biotransformation, mainly glucuronidation. Urinary elimination of glucuronide conjugates is a major route of NVP disposition (Riska et al., 1999a).

Several CYP isoforms participate in NVP biotransformation into its phase I metabolites (Erickson et al., 1999). The formation of 2-OH-NVP is mediated by CYP3A, while 3-OH-NVP formation is attributed to CYP2B6. CYP3A4, CYP2B6, and CYP2D6 are involved in 8-OH-NVP formation. The formation of 12-OH-NVP, the main NVP phase I metabolite, is mediated by CYP2A6, CYP3A4, CYP3A5, CYP2D6, CYP2C9 and also CYP2C19. Finally, 4-COOH-NVP is formed by secondary oxidation of 12-OH-NVP (Erickson et al., 1999, Riska et al., 1999a, Chen et al., 2008).

NVP induces its own metabolism through an inductor effect on CYP3A4 and CYP2B6; this auto-induction effect is complete within 28 days and then NVP plasma levels reach the steady-state (Lamson et al., 1999, Riska et al., 1999a).

### 1.2.2. Nevirapine toxicology: the role of bioactivation

Despite its efficacy and clinical advantages, NVP is associated with severe hepatotoxic reactions and skin rash. These adverse events may lead to drug discontinuation or even be fatal (Pollard et al., 1998, Cattelan et al., 1999, FDA, 2000, Taiwo, 2006, De Lazzari et al., 2008). Moreover, epidemiological data suggest an association between chronic NNRTI use and an increased incidence of non-AIDS-defining cancers in HIV-infected patients (Powles et al., 2009).

Increasing evidence has supported a higher risk of NVP-induced toxicity in women and in those individuals with higher $CD4^+$ cell count (Ho et al., 1998, Antinori et al., 2001, Bersoff-Matcha et al., 2001, De Lazzari et al., 2008, Kiertiburanakul et al., 2008, Medrano et al., 2008). Thus, it is recommended that NVP should be initiated in HIV-infected women with a $CD4^+$ count below 250 cells/µL. This cutoff is higher for men, who should initiate NVP-containing cART with a $CD4^+$ count below 400 cells/µL (Thompson et al., 2010). Besides female sex and immunocompetence, other risk factors have been identified such as Asian ethnicity (Ho et al., 1998, Ananworanich et al., 2005),

detectable viral load (Kesselring et al., 2009), low body mass index and history of drug allergy (Kiertiburanakul et al., 2008).

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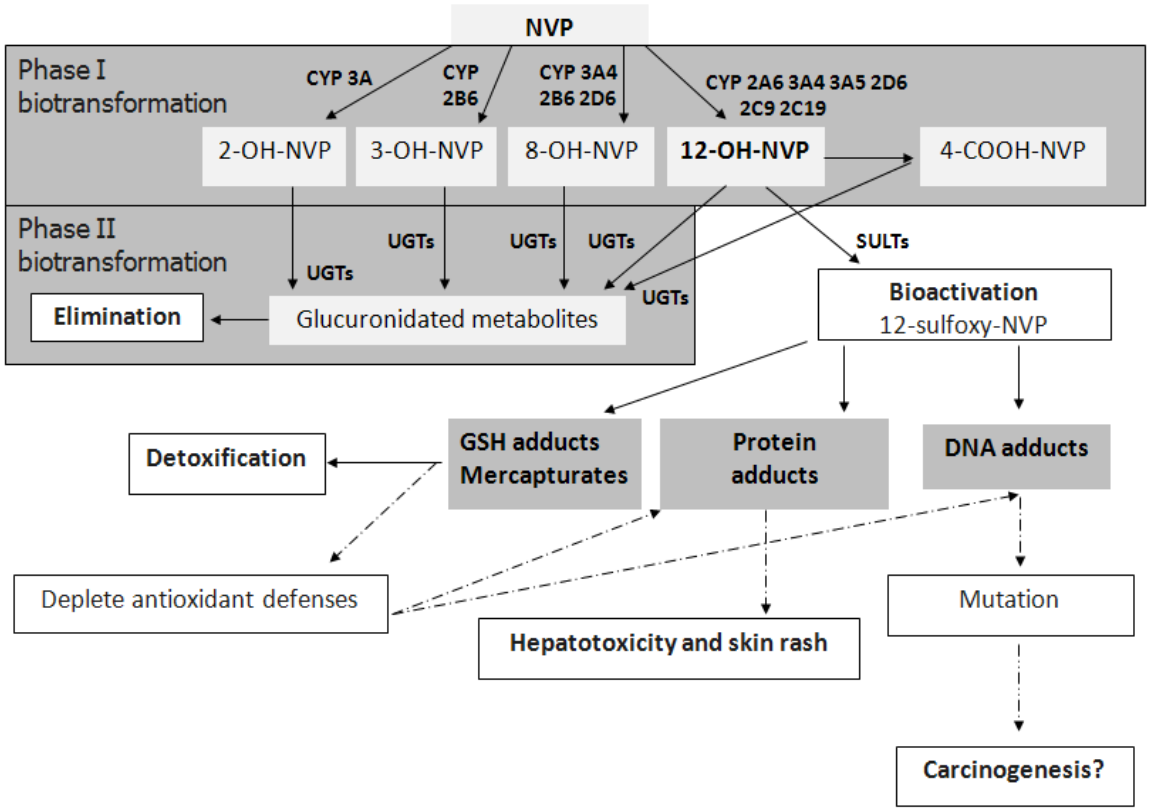


**Figure 3 Nevirapine biotransformation, disposition and proposed bioactivation pathways.** Nevirapine (NVP) is metabolized by several isoforms of cytochrome P450 (CYP) yielding several phase I metabolites (Riska et al., 1999a): 2-hydroxy-nevirapine (2-OH-NVP), 3-hydroxy-nevirapine (3-OH-NVP), 8-hydroxy-nevirapine (8-OH-NVP), and 12-hydroxy-nevirapine (12-OH-NVP). The 12-OH-NVP metabolite is further oxidized by CYP450 yielding 4-carboxy-nevirapine (4-COOH-NVP) (Chen et al., 2008). The phase I NVP metabolites undergo extensive glucuronidation, which represents a major pathway of NVP elimination (Riska et al., 1999a). The bioactivation of 12-OH-NVP by sulfotransferases (SULTs) can generate 12-sulfoxy-NVP, a reactive metabolite that binds covalently to proteins and DNA (Antunes et al., 2008, Caixas et al., 2012, Meng et al., 2013). The formation of DNA adducts could explain the increased incidence of non-AIDS defining cancers among HIV-infected patients treated with non-nucleoside reverse transcriptase inhibitors (Powles et al., 2009). Also, the formation of adducts with proteins could explain the NVP-associated adverse reactions, hepatotoxicity and skin rash (Yuan et al., 2011). The presence of glutathione (GSH) adducts and mercapturates in patients and animal models treated with NVP has also been detected (Srivastava et al., 2010a).

Even though the exact mechanisms underlying the idiosyncratic toxicity of NVP remain partially unknown, increasing evidence has supported the role of bioactivation on the onset of NVP-associated toxicity (Antunes et al., 2008, Chen et al., 2008, Antunes et al., 2010a, Antunes et al., 2010b, Srivastava et al., 2010a, Caixas et al., 2012, Pereira et al., 2012a, Pereira et al., 2012b, Meng et al., 2013, Sharma et al., 2013b, Sharma et al., 2013a).

Moreover, besides CYP mediated-metabolism of NVP yield several phase I metabolites which undergo to extensive glucuronidation (Riska et al., 1999a), the involvement of other phase II metabolic pathways (Figure 3), namely sulfotransferases (SULTs) should not be excluded.

SULTs-mediated biotransformation is a process of critical importance in the metabolism of xenobiotics (Gamage et al., 2006). These reactions are involved in detoxification of several compounds (Glatt, 2000, McGill and Jaeschke, 2013), however these phase II enzymes may be also involved in the bioactivation of toxic and mutagenic xenobiotics, with 3'-phosphoadenosine-5'-phosphosulfate (PAPS) playing a crucial role in these processes, as SULTs' cofactor (Boocock et al., 2000, Glatt, 2000, Suzuki et al., 2012). PAPS is the universal donor of the sulfate moiety, which can be transferred to an acceptor molecule (Gamage et al., 2006). PAPS is biosynthesized in two steps: the first reaction is catalyzed by ATP-sulfurylase, yielding adenosine 5'-phosphosulfate (APS), the subsequent reaction is promoted by APS kinase and results in the formation of PAPS (Venkatachalam et al., 1998, Fuda et al., 2002). The biosynthesis of PAPS in humans is carried out by a single enzyme with both ATP-sulfurylase and APS kinase activities, named PAPS synthase (Venkatachalam et al., 1998).

Indeed, Chen *et al* (2008) have firstly showed the formation of a sulfate of 12-OH-NVP in the bile and urine samples of Brown Norway rats with NVP-induced skin rash.

Moreover, Antunes and her team (Antunes et al., 2008, Antunes et al., 2010a, Antunes et al., 2010b), using 12-mesiloxy-NVP as a synthetic surrogate of 12-sulfoxy-NVP, showed the *in vitro* reactivity of this electrophilic derivative with several aminoacids (Antunes et al., 2010a), with human serum albumin and haemoglobin (Antunes et al., 2010b) and also with nucleosides and DNA (Antunes et al., 2008).

In addition, Srivastava *et al* (2010a) have identified two structurally distinct mercapturates, substituted at C3 and C12 positions, in urine of animal models exposed to

NVP and also in urine of HIV-infected patients. Mercapturates have been classically associated to exposure to reactive electrophiles (Hinchman and Ballatori, 1994).

Recently, our group (Caixas et al., 2012) showed, for the first time, the formation of 12-OH-NVP-derived haemoglobin adducts in HIV-infected patients. Also, Meng *et al* (2013) showed the formation of NVP-human serum albumin adducts, formed upon reaction with 12-sulfoxy-NVP.

Additionally, Sharma *et al* (2013a) showed the covalent binding of 12-sulfoxy-NVP with proteins after incubation of this sulfate with skin homogenate. These covalent modification of skin proteins only occur in the presence of PAPS, which supports the role of 12-sulfoxy-NVP on protein adduct formation and the role of SULTs in 12-OH-NVP bioactivation.

The formation of adducts with proteins might explain the NVP-induced adverse reactions, since the covalent modification of proteins can alter their normal function or trigger an immune response against the modified protein, potentially leading to mitochondrial injury and cell death (Russmann et al., 2009). Also, the formation of adducts with DNA could explain the epidemiological evidence presented by Powles *et al* (2009) of an association between the incidence of non-AIDS defining cancers among HIV-infected patients and the antiretroviral treatment with NNRTIs.

In addition, other reactive metabolites have been proposed, such as a quinone methide possibly generated from 12-sulfoxy-NVP (Chen et al., 2008). However, the formation of this reactive quinone may probably occur in the absence of phase II reactions, by CYP-mediated dehydrogenation of NVP or by dehydration of 12-OH-NVP (Wen et al., 2009). Also, a reactive quinone-imine was found to be derived from *in vitro* chemical and enzymatic oxidation of 2-OH-NVP (Antunes et al., 2011, Pereira et al., 2012a). This evidence supports the hypothesis that other metabolites, besides 12-OH-NVP, may have an important role in NVP toxicity.

Another aspect of critical importance is the influence of HIV infection and AIDS status in bioactivation of drugs and detoxification of reactive species (Esteban et al., 1997). For instance, HIV infection itself is associated with impaired synthesis and lower levels of glutathione (GSH) (Smith et al., 1996), and also with increased lipid peroxidation (Wanchu et al., 2009, Awodele et al., 2012, Morris et al., 2012), with patients under persistent oxidative and inflammatory states. Even more, depleted levels of GSH has been

associated with HIV disease progression (Herzenberg et al., 1997). This depletion of GSH could avoid an efficient detoxification of reactive metabolites, which will be available for reacting with macromolecules, such as proteins or DNA (Pereira et al., 2012a). Moreover, Adaramoye *et al* (2012) reported a significant and dose-dependent increase of lipid peroxidation levels in liver, kidney and testis of animal models exposed to NVP. These authors also showed that NVP administration causes a decrease in the activity of antioxidant enzymes in these organs (Adaramoye et al., 2012). Claes *et al* (2004 ) had provided indirect evidence supporting the importance of GSH levels in the elimination of toxic NVP metabolites. These authors reported the recovery of an HIV-infected patient suffering from NVP-induced hepatotoxicity and toxic epidermal necrolysis, after treatment with intravenous immunoglobulins and N-acetylcysteine, a GSH precursor.

### 1.2.3. Nevirapine pharmacogenetics

The association between NVP-induced adverse events and higher $CD4^+$ lymphocyte counts supports a role for an immune-mediated reaction on NVP toxicity, and two alternative pathways have been suggested (Yuan et al., 2011): hepatotoxic reactions seem to be mediated by major histocompatibility complex (MHC) class II whereas cutaneous toxic reactions are probably mediated by MHC class I. Indeed, there are several studies that have associated human leukocyte antigen (HLA) polymorphisms with increased risk of NVP-related toxicity. For instance, HLA-B*3505 and HLA-Cw*0401 alleles have been associated with an increased risk of NVP-induced skin rash (Chantarangsu et al., 2009, Likanonsakul et al., 2009, Arab-Alameddine et al., 2011, Yuan et al., 2011). In a cohort of HIV-infected Chinese Han the presence of HLA-Cw*04 was a risk factor for NVP-associated hypersensitivity (Gao et al., 2012), and the presence of HLA-Cw*0401 was recently associated with higher risk of NVP-related *Stevens Johnson* Syndrome and toxic epidermal necrolysis, in a Malawian HIV cohort (Carr et al., 2013). Moreover, an association between HLA-DRB1*0101 and NVP-induced liver toxicity has been reported (Martin et al., 2005 , Arab-Alameddine et al., 2011, Yuan et al., 2011). HLA-Cw8 and HLA-B14 were also associated with an increased risk of NVP-induced hypersensitivity among Sardinians patients (Littera et al., 2006).

Besides genetic variations on HLA, other genes may be implicated on NVP-associated adverse events. Data from a genome-wide association study showed that variations in the coiled-coil alpha-helical rod protein 1 gene (CCHCR1) is probably associated with NVP-induced skin rash (Chantarangsu et al., 2011). Alterations on CCHCR1 gene are also seen in psoriasis (Tiala et al., 2008) and skin cancer (Suomela et al., 2009).

Moreover, polymorphisms on CYP metabolizing enzymes can alter NVP metabolite profile and pharmacokinetics, drug clearance, its efficacy and toxicity (Michaud et al., 2012). Saitoh *et al* (2007) have showed the influence of CYP2B6 G516T on NVP pharmacokinetics in a pediatric cohort; these authors have demonstrated that children who carried TT genotype have decreased oral clearance of NVP. Indeed, there is evidence of the association between this genotype and a significantly higher NVP plasma levels and lower clearance (Wyen et al., 2008, Mahungu et al., 2009, Chou et al., 2010, Calcagno et al., 2012). Also, CYP2B6 T983C is associated with higher NVP plasma levels (Wyen et al., 2008, Calcagno et al., 2012, Heil et al., 2012). Regarding polymorphisms on CYP2C19, preliminary data of our team have recently pointed towards no influence on 12-OH-NVP formation. Our group did not find any differences in NVP:12-OH-NVP ratio when comparing CYP2C19*17 (ultra-rapid metabolizer), CYP2C19*2 (poor metabolizer) and CYP2C19*1 (wild type) (Grilo et al., 2013).

The presence of polymorphisms in drug transporters may also alter NVP pharmacokinetics (Michaud et al., 2012). Single nucleotide polymorphism (SNP) rs2125739 in the ABCC10 transporter gene was recently associated with NVP plasma concentration, being more prevalent among patients with lower NVP plasma levels (Liptrott et al., 2012). Contrariwise, the SNPs on ABCB1 (C3435T and C1236T) are not associated with any effect on NVP pharmacokinetic profile (Calcagno et al., 2012, Uttayamakul et al., 2012). However, in case-control studies (patients with NVP-associated hepatotoxicity vs. patients without any toxic reaction) the variant T allele of ABCB1 C3435T SNP showed a protective effect against the development of liver injury (Haas et al., 2006, Ritchie et al., 2006, Ciccacci et al., 2010).

## 1.3. Sex differences on biotransformation and adverse drug reactions: are women at increased risk?

Sex-related differences in pharmacokinetics is an issue of growing interest. Increasing evidence has shown that sex can influence several aspects of drug pharmacokinetics, namely phase I (Lamba et al., 2003, Hirao et al., 2011, Baggio et al., 2013, Franconi and Campesi, 2013 ) and II biotransformation (Tsoi et al., 2001, Wu et al., 2001, Alnouti and Klaassen, 2006, Gallagher et al., 2010, Hirao et al., 2011, Suzuki et al., 2012). For instance, Lamba et al (2003), in a study carried out with human liver microsomes, reported higher hepatic expression, higher amount of protein and also higher activity of CYP2B6 among women, and also a lower frequency of poor CYP2B6 metabolizers among them. Also, women have higher expression and activity of hepatic CYP3A4 (Parkinson et al., 2004, Waxman and Holloway, 2009, Baggio et al., 2013, Franconi and Campesi, 2013 ), being the amount of CYP3A4 enzyme among women at least twice than men (Parkinson et al., 2004). Furthermore, regarding phase II biotransformation, it is well documented the influence of sex on activity and expression of SULTs, PAPS synthase and UDP-glucuronosyltransferases (Tsoi et al., 2001, Wu et al., 2001, Alnouti and Klaassen, 2006, Gallagher et al., 2010, Hirao et al., 2011, Suzuki et al., 2012). These differences might be at least partially explained by sex differences in the secretion pattern of hormones, such as androgens, oestrogens and growth hormone (Alnouti and Klaassen, 2011). Also, the frequency and severity of adverse drug reactions are generally higher in women (Martin et al., 1998, Hellden et al., 2009, Franconi and Campesi, 2013 , Miguel et al., 2013 ). In addition, female sex is associated with a higher incidence of autoimmune diseases (Mallampalli et al., 2013, Tiniakou, 2013), and with a stronger humoral and cellular immune response (Voskuhl, 2011). These differences may also contribute to a dimorphic profile of NVP-related adverse events, with women being at higher risk. As mentioned previously, the current evidence is strongly consistent with a higher risk in women (Ho et al., 1998, Antinori et al., 2001, Bersoff-Matcha et al., 2001) and with a subjacent immune-mediation (Martin et al., 2005 , Taiwo, 2006, Yuan et al., 2011, Dong et al., 2012).

Increasing evidence has emerged regarding the importance of NVP biotransformation to the onset of its toxicity (Caixas et al., 2012, Meng et al., 2013, Sharma et al., 2013a,

Sharma et al., 2013b); these new insights highlight the importance of exploring sex-related variations in NVP toxic mechanism. A better understanding of these differences is essential and might lead to more appropriate risk assessment and to the development of safer therapeutic strategies for HIV-infected women exposed to NVP-based cART.

## 1.4. Rational and objectives

What we know:

- NVP is a widely used antiretroviral drug for the treatment of HIV-1 infection;
- Despite NVP efficacy in the suppression of viral replication, the chronic use of this drug has been associated with severe, and even life-threatening, liver and skin toxicity;
- Women are at increased risk for developing NVP-induced toxic events;
- NVP biotransformation is a process of utmost importance to the onset of its toxicity, through the formation of reactive metabolites.

Disparities in NVP biotransformation and generation of toxic metabolites between men and women might be the underlying cause. Thus, the present work was aimed to:

- Develop and validate a high-performance liquid chromatography (HPLC) methodology to quantify NVP and its main phase I metabolites (2-OH-NVP, 3-OH-NVP, 8-OH-NVP and 12-OH-NVP);
- Explore sex differences in NVP biotransformation as a potential factor in NVP-induced toxicity.

# 2.Materials and Methods

## 2.1. Development and validation of an analytical method

### 2.1.1. Reagents

Water was filtered using a *Millipore Milli-Q* Water Purification System (Billerica, MA). The 2-OH-NVP, 3-OH-NVP, 8-OH-NVP and 12-OH-NVP metabolites were synthesized as described by Grozinger et al (2000) and Antunes et al (2011) and were used as standards for identification and quantification purposes. NVP was obtained from *Cipla* (Maharashtra, India). Dichloromethane, acetonitrile and methanol were supplied by *VWR* (Radnor, PA), dimethylsulfoxide was purchased from *Mallinckrodt Baker* (Center Valley, PA) and ammonium acetate was obtained from *Merck KGaA* (Darmstadt, Germany).

### 2.1.2. High-performance liquid chromatography conditions

Separation of NVP and its phase I metabolites by HPLC was performed in an Agilent 1100 Series system (*Agilent Technologies*, Santa Clara, CA, USA) using a reverse-phase Luna C18 column (250 mm × 4.6 mm; 5 μm; 100 Å; *Phenomenex*, Torrance, CA, USA). The mobile phase – 10% acetonitrile in 15 mM ammonium acetate buffer, pH 4 – was delivered at a flow rate of 0.8 mL/min for 90 minutes; the flow rate was then increased to 1.5 mL/min in 5 minutes and maintained at this value for an additional period of 19 minutes. The column temperature was 40ºC, the injection volume was 100 μL, and UV absorbance was monitored at 254 nm.

### 2.1.3. Preparation of stock standard solutions

Stock solutions of NVP, 3-OH-NVP, 8-OH-NVP and 12-OH-NVP were prepared in methanol, at a concentration of 1 mg/mL, from four distinct weighings of each analyte. The stock solutions of 2-OH-NVP were prepared in 12.5% dimethylsulfoxide in methanol at a concentration of 1 mg/mL. These stock solutions were then diluted to 0.1 mg/mL for use in the preparation of standard samples for calibration curve and quality control (QC).

These solutions were stored at -80ºC.

### 2.1.4. Preparation of samples for calibration curve and quality control

Samples for calibration curve of metabolites were prepared by successive dilutions to span the following concentrations of each metabolite in plasma: 2,500, 1,000, 500, 250, 100, 50, 25 and 10 ng/mL. Samples for NVP calibration curve were prepared in the same manner, spanning concentrations in plasma of 10,000, 5,000, 2,500, 1,000, 500, 250, 100, 50, 25 and 10 ng/mL.
QC samples were prepared from a distinct stock solution at a concentration of 0.1 mg/mL, by successive dilutions to obtain a final concentration in plasma of 1,250, 200, 20 (QC1, QC2 and QC3, respectively) and 10 ng/mL.

### 2.1.5. Extraction of analytes from plasma

Aliquots of the samples (900 µL) were heated at 60ºC for 60 min before handling at room temperature. Analytes were then extracted from plasma with dichloromethane; the organic phase was dried under vacuum and the dried residue was reconstituted in 150 µL of a 1:1 methanol :water solution.

### 2.1.6. Linearity of the method

Three calibration curves were prepared from different stock solutions. Calibration standards ranged 10 to 10,000 ng/ml for NVP and 10 to 2,500 ng/mL for NVP metabolites. The calibration curves were plotted by linear regression of the chromatographic peak area (mAU*min) as a function of analytes concentration (ng/mL) to assess the linearity of the method. The average back-calculated concentrations were also assessed.

### 2.1.7. Lower limit of quantitation

The lower limit of quantitation (LLOQ) was defined as the lowest concentration of each analyte which could be accurately and reproducibly quantified. In order to validate the LLOQ, 12 samples with a concentration of 10 ng/mL were analyzed for the accuracy and inter-assay precision assessment and 5 samples were analyzed for the intra-assay precision assessment.

### 2.1.8. Selectivity and carry-over

In order to minimize interference from endogenous plasma compounds, calibration standards and QC samples were prepared by spiking a pool of plasma from healthy volunteers with the analytes. Also, a blank sample, without any analyte, was prepared and extracted as previously described in each HPLC analysis.

In addition, and taking into account that NVP is administered as part of cART, the interference of other commonly administered antiretroviral drugs with NVP and its metabolites was also evaluated.

For the study of the carry-over effect, two blank samples were prepared and injected into the HPLC system after the analysis of the most concentrated standard calibration sample.

### 2.1.9. Accuracy

Accuracy was defined as the closeness to theoretical concentration of the QC samples and was calculated as the ratio between the measured and theoretical concentrations, expressed in percentage.

Three concentrations not evaluated in the linearity assessment, but included within the calibration range (QC1, QC2 and QC3), were used in three validation runs. Accuracy was also evaluated for LLOQ.

The mean concentration of each QC sample analyzed should be within 85 and 115% of the theoretical concentration, except for the LLOQ, for which 80 and 120% of the

theoretical concentration is acceptable (FDA, 2001, EMA, 2011).

### 2.1.10. Intra-assay and inter-assay precision

Assay precision was defined as the concordance between multiples measures. Intra-assay and inter-assay precision were assessed by analysis of the QC samples, QC1, QC2, QC3 and the LLOQ.

Intra-assay precision was assessed by the coefficient of variation (CV) obtained from the analysis of QC samples in five analytical runs on the same day. Inter-assay precision was also assessed from the CV obtained from QC and LLOQ samples, evaluated in different days.

The intra-assay and inter-assay CV should not exceed 15%, except for LLOQ for which 20% of variation is acceptable (FDA, 2001, EMA, 2011).

### 2.1.11. Recovery

Recovery was assessed by comparing measurements obtained from QC samples prepared in plasma with those obtained with solutions of the analytes prepared at the same concentrations in water instead of plasma. The recovery assay was performed for QC1, QC2 and QC3.

### 2.1.12. Statistical analyses

Statistical analysis was performed using GraphPad Prism version 5.0 (*Graph Pad Software Inc.,* San Diego, CA, USA). The data are presented in percentage, mean and standard error of the mean (SEM).

## 2.2. Exploring sex differences on nevirapine biotransformation in HIV-infected patients

### 2.2.1. Ethical issues, patients inclusion, data gathering and blood sampling

The current work was conducted in accordance with the Declaration of Helsinki. The study protocol received prior approval from the Ethics Committees of *Centro Hospitalar de Lisboa Central, EPE* (process number 32-CHLC) and *Hospital Prof. Doutor Fernando Fonseca, EPE* (process number CA21/2011), and was also approved by the National Committee for Data Protection (process number 6567/2009). The patients gave their written informed consent and adherence was controlled by the clinician.

All patients were adults with documented HIV infection who had been using NVP-containing cART (400 mg once daily) for at least 1 month, regardless of the past therapeutic history. Exclusion criteria were being < 18 years of age, having AIDS-defining conditions, and compliance issues.

The following data were gathered for each patient: age, ethnicity, weight, height, time on NVP, time between last NVP intake and blood sampling, viral load, $CD4^+$ cell count and hepatic function biomarkers [alanine amino-transferase (ALT), lactate dehydrogenase (LDH), gamma-glutamyltransferase (GGT), alkaline phosphatase (ALKP) and bilirubin]. Blood samples (2 mL) were collected into EDTA-containing tubes.

### 2.2.2. Extraction of analytes from blood samples and HPLC quantification

Plasma was obtained by centrifugation of blood at 3000 g for 10 min. Aliquots of the plasma (900 µL) were heated at 60 ºC for 60 min for viral inactivation before handling at room temperature.

Analytes were then extracted as described in *Section 2.1.5*. The plasma levels of NVP and its metabolites were quantified as described in *Section 2.1.2*.

### 2.2.3. Statistical analyses

Systemic exposure to the different NVP metabolites was assessed in terms of absolute concentrations and as the proportion of each metabolite in the plasma. Mean (± SEM), median (IQR) and frequencies (%) were used to describe the study population. *Student's t*-test was performed for comparisons between means and the *Mann-Whitney U*-test was performed for comparisons between medians. The statistical analyses were performed using GraphPad Prism version 5.0.

# 3. Results

## 3.1. Development and validation of an analytical method

### 3.1.1. Chromatographic separation of the analytes

Using an isocratic elution with 10% acetonitrile in 15 mM ammonium acetate buffer, pH 4, on a C18 reversed phase column (cf. *Section 2.1.2.*), the chromatographic peaks occurred at 34 min for 2-OH-NVP, 52 min for 12-OH-NVP, 69 min for 3-OH-NVP, 85 min for 8-OH-NVP and 109 min for NVP (Figure 4).

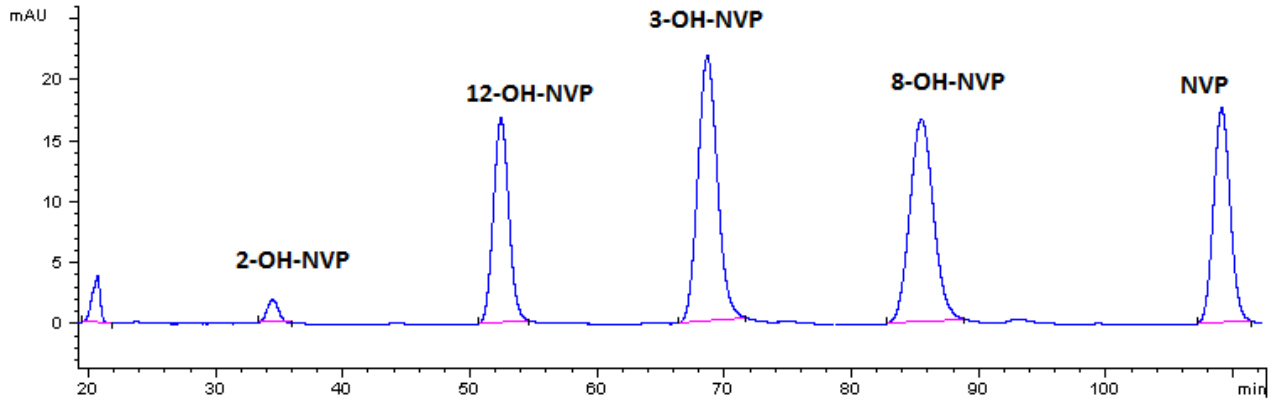


**Figure 4. Representative chromatogram of a mixture of nevirapine (NVP) and its phase I metabolites under the conditions described in this study.** The analyte concentrations were 2,500 ng/mL.

### 3.1.2. Linearity of the method

The evaluation of linearity was performed using calibration standards ranged from 10 to 2,500 ng/mL for the NVP metabolites and 10 to 10,000 ng/mL for NVP. The calibration curves for NVP and its metabolites were obtained using 10 (for NVP) and 8 (for each metabolite) standard samples.

The linear regression model showed to be the most suitable for fitting a function to the points obtained (*p* value of *Runs Tests* > 0.05). It was demonstrated that the concentration of standard samples significantly influences the chromatographic signal area (*p* value of *F tests* < 0.0001) for each analyte. The coefficient of determination, $r^2$, was > 0.99 for all

analytes.

For all calibration curves with the exception for 2-OH-NVP curve, the 95% confidence interval for intercept contained zero. For 2-OH-NVP, although the 95% confidence interval did not contain zero, the value of intercept was small (1 ± 0.3 mAu*min).

The average back-calculated concentrations for NVP and its metabolites was also evaluated (Table 2). The CV were lower than 9% for all analytes.

**Table 2 Average back-calculated calibration standards**

| Theoretical concentration (ng/mL) | CV (%) | | | | |
|---|---|---|---|---|---|
| | **NVP** | **2-OH-NVP** | **3-OH-NVP** | **8-OH-NVP** | **12-OH-NVP** |
| 10,000 | 0.9 | - | - | - | - |
| 5,000 | 4.2 | - | - | - | - |
| 2,500 | 2.5 | 0.2 | 0.5 | 0.9 | 0.2 |
| 1,000 | 0.3 | 1.6 | 2.6 | 4.9 | 0.8 |
| 500 | 0.8 | 0.1 | 1.6 | 1.9 | 0.7 |
| 250 | 3.8 | 0.5 | 2.7 | 2.0 | 0.5 |
| 100 | 6.6 | 8.5 | 6.3 | 6.3 | 3.2 |
| 50 | 6.6 | 1.5 | 3.7 | 2.1 | 2.5 |
| 25 | 1.7 | 1.6 | 0.9 | 0.2 | 1.0 |
| 10 | 3.1 | 7.6 | 4.6 | 1.9 | 6.6 |

NVP, nevirapine; 2-OH-NVP, 2-hydroxy-nevirapine; 3-OH-NVP, 3-hydroxy-nevirapine; 8-OH-NVP, 8-hydroxy-nevirapine; 12-OH-NVP, 12-hydroxy-nevirapine.

### 3.1.3. Lower limit of quantitation

The LLOQ of the current method was 10 ng/mL for each analyte. The CV of multiples measures of each standard at a concentration of 10 ng/mL was lower than 17% for all analytes. Although a standard at a concentration of 5 ng/mL was also tested, the chromatographic peaks were not distinguishable from background noise at this concentration.

### 3.1.4. Selectivity and carry-over effect

The analysis of blank plasma samples showed no interferences from endogenous compounds for any of the analytes (Figure 5). Also, no interference from other commonly administered antiretrovirals was observed: tenofovir, lamivudine, didanosine, emtricitabine, zidovudine and abacavir eluted at 4.5, 5.1, 5.8, 6.7, 21.9, and 22.5 min, respectively, under the same elution conditions (not shown). Ritonavir and lopinavir eluted after NVP, in the post-run period.

No carry-over effect was observed when two blank samples were injected in the HPLC system after analysis of the most concentrated calibration standard sample.

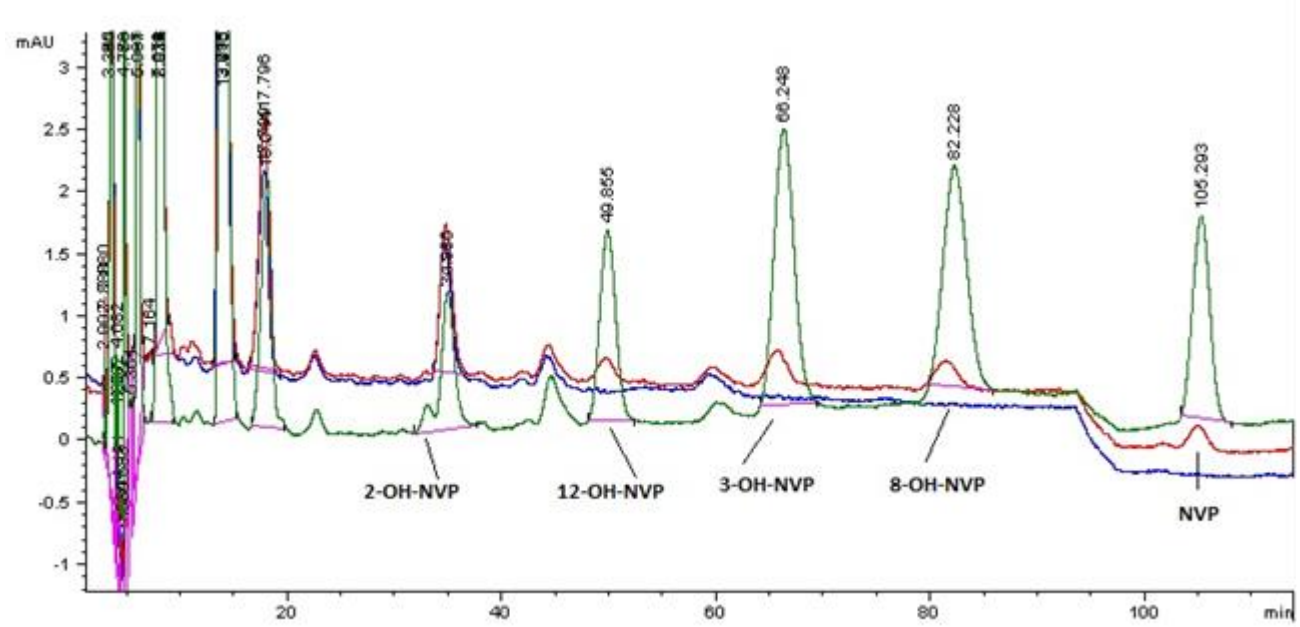


**Figure 5. Representative analytical run chromatograms**: a blank plasma sample (blue line), a standard containing 250 ng/mL of each analyte (green line), and a standard containing 10 ng/mL of each analyte (red line).

### 3.1.5. Accuracy, precision and recovery

The results of the accuracy, intra-assay and inter-assay precision are presented in Table 3 and 4, respectively.

The accuracy ranged from 90 to 120% at the LLOQ and from 85 to 112% for QC samples (Table 3).

**Table 3 Results obtained in the accuracy assay**

| Analyte | Concentration of quality control samples (ng/mL) | Accuracy (%) |
|---|---|---|
| NVP | 10 | 120 |
| | 20 | 101 |
| | 200 | 92 |
| | 1,250 | 88 |
| 2-OH-NVP | 10 | 91 |
| | 20 | 85 |
| | 200 | 109 |
| | 1,250 | 109 |
| 3-OH-NVP | 10 | 100 |
| | 20 | 98 |
| | 200 | 107 |
| | 1,250 | 95 |
| 8-OH-NVP | 10 | 102 |
| | 20 | 109 |
| | 200 | 111 |
| | 1,250 | 101 |
| 12-OH-NVP | 10 | 108 |
| | 20 | 101 |
| | 200 | 99 |
| | 1,250 | 92 |

NVP, nevirapine; 2-OH-NVP, 2-hydroxy-nevirapine; 3-OH-NVP, 3-hydroxy-nevirapine; 8-OH-NVP, 8-hydroxy-nevirapine; 12-OH-NVP, 12-hydroxy-nevirapine.

The results of intra-assay precision were between 5 to 16% at the LLOQ and between 4 to 15% for all other QC samples. The values of inter-assay precision ranged from 10 to 17% at the LLOQ and from 7 to 15% for QC1, QC2 and QC3 (Table 4).
A liquid-liquid extraction protocol allowed a mean recovery of 94% (Table 5).

**Table 4 Values of intra-assay and inter-assay precision**

| Analyte | Concentration of quality control samples (ng/mL) | Intra-assay precision | Inter-assay precision |
|---|---|---|---|
| | | CV (%) | CV (%) |
| NVP | 10 | 6 | 17 |
| | 20 | 11 | 11 |
| | 200 | 6 | 8 |
| | 1,250 | 6 | 7 |
| 2-OH-NVP | 10 | 15 | 12 |
| | 20 | 14 | 13 |
| | 200 | 5 | 9 |
| | 1,250 | 15 | 11 |
| 3-OH-NVP | 10 | 12 | 11 |
| | 20 | 10 | 14 |
| | 200 | 4 | 9 |
| | 1,250 | 6 | 8 |
| 8-OH-NVP | 10 | 7 | 13 |
| | 20 | 7 | 8 |
| | 200 | 6 | 9 |
| | 1,250 | 5 | 8 |
| 12-OH-NVP | 10 | 6 | 11 |
| | 20 | 9 | 14 |
| | 200 | 5 | 8 |
| | 1,250 | 6 | 7 |

CV, coefficient of variation; NVP, nevirapine; 2-OH-NVP, 2-hydroxy-nevirapine; 3-OH-NVP, 3-hydroxy-nevirapine; 8-OH-NVP, 8-hydroxy-nevirapine; 12-OH-NVP, 12-hydroxy-nevirapine.

**Table 5 Recovery assessment**

| Analyte | Concentration of quality control samples (ng/mL) | Recovery (%) |
|---|---|---|
| NVP | 20 | 93 |
| | 200 | 93 |
| | 1,250 | 100 |
| 2-OH-NVP | 20 | 74 |
| | 200 | 100 |
| | 1,250 | 99 |
| 3-OH-NVP | 20 | 81 |
| | 200 | 99 |
| | 1,250 | 93 |
| 8-OH-NVP | 20 | 100 |
| | 200 | 100 |
| | 1,250 | 85 |
| 12-OH-NVP | 20 | 94 |
| | 200 | 96 |
| | 1,250 | 99 |

NVP, nevirapine; 2-OH-NVP, 2-hydroxy-nevirapine; 3-OH-NVP, 3-hydroxy-nevirapine; 8-OH-NVP, 8-hydroxy-nevirapine; 12-OH-NVP, 12-hydroxy-nevirapine.

## 3.2. Exploring sex differences on nevirapine biotransformation in HIV-infected patients

A total of 52 patients (63% men) were included in the study. The patients' anthropometric and clinical data are presented in Table 6. Body weight was significantly higher in men compared with women, but the body mass index was similar in the two groups. Among the liver function tests analyzed, differences were found for LDH and ALKP, which were higher in women. There were no differences between sexes regarding age, time on NVP,

time between sampling and last NVP intake, $CD4^+$ cell counts, ALT, GGT and direct bilirubin; the total bilirubin concentration was higher in men (Table 6). All patients had an undetectable viral load.

**Table 6 Patients' anthropometric and clinical data.**

| Parameters | Men | Women | *p* value |
|---|---|---|---|
| N | 33 | 19 | |
| Proportion of non-Caucasians (%) | 42 | 32 | |
| Age (years) [a] | 50 [39-60] | 46 [38-63] | NS |
| Body weight (kg) [b] | 73 ± 2 | 65 ± 3 | 0.028 |
| BMI ($kg/m^2$) [b] | 25 ± 1 | 25 ± 1 | NS |
| Time on NVP (years) [a] | 4 [2-9] | 4 [2-7] | NS |
| Time between sampling and last NVP intake (h) [a] | 12 [6-15] | 13 [11-15] | NS |
| $CD4^+$ cell count ($cells/mm^3$) [a] | 515 [386-675] | 575 [413-735] | NS |
| ALT (U/L) [a] | 36 [23-47] | 31 [26-45] | NS |
| ALKP (U/L) [a] | 80 [69-106] | 110 [82-155] | 0.036 |
| LDH (U/L) [a] | 181 [167-243] | 228 [196-244] | 0.037 |
| GGT (U/L) [a] | 70 [36-134] | 59 [51-126] | NS |
| Total Bilirubin(mg/dL) [a, c] | 0.39[0.33-0.47] (n=25) | 0.33[0.27-0.40] (n=15) | 0.026 |
| Direct Bilirubin (mg/dL) [a, c] | 0.1 [0.08-0.11] (n=17) | 0.1 [0.07-0.11] (n=13) | NS |

ALKP, alkaline phosphatase; ALT, alanine amino-transferase; BMI, body mass index; GGT, gamma-glutamyltransferase; LDH, lactate dehydrogenase; NS, not significant; NVP, nevirapine.
[a] *Mann-Whitney U* Test, Median [IQR]
[b] *Student's t*-Test, Mean ± SEM
[c] The missing values correspond to patients for whom clinical data were not available.
Differences were considered significant if $p < 0.05$.

Sex differences in the plasma concentration of NVP and in its metabolite profile are presented in Table 7. Women had higher NVP and 3-OH-NVP plasma concentrations, when adjusted for body weight. All patients had 8-OH-NVP levels below the LLOQ of

our method. Sex differences in the proportions of NVP metabolites are presented in Table 8. Women had a significantly higher proportion of 12-OH-NVP and 3-OH-NVP compared with men, when the data were adjusted for body weight.

**Table 7 Plasma concentration levels of nevirapine and its phase I metabolites.**

| | | **Men** | **Women** | ***p* value** |
|---|---|---|---|---|
| | N | 33 | 19 | |
| Analytes (ng/mL) | NVP [a] | 4279 [2678-5105] | 4233 [3533-5130] | NS |
| | 2-OH-NVP [a, b] | 58.6[29.5-109.6] (n=19) | 63.2 [31.3-80.5] (n=7) | NS |
| | 3-OH-NVP [a, b] | 24.0 [17.2-31.3] (n=31) | 33.7 [22.7-38.6] (n=17) | 0.047 |
| | 12-OH-NVP [a] | 364.9 [247.3-543.4] | 371.0 [270.2-450.7] | NS |
| Analytes (ng/mL/kg) | NVP [a] | 53.9 [34.6-72.9] | 70.7 [51.8-86.4] | 0.030 |
| | 2-OH-NVP [a, b] | 0.73 [0.42-1.78] (n=19) | 0.92 [0.34-1.73] (n=7) | NS |
| | 3-OH-NVP [a, b] | 0.34 [0.23-0.47] (n=31) | 0.52 [0.32-0.73] (n=17) | 0.035 |
| | 12-OH-NVP [a] | 4.80 [3.48-8.47] | 6.06 [3.87-7.79] | NS |

2-OH-NVP, 2-hydroxy-nevirapine; 3-OH-NVP, 3-hydroxy-nevirapine; 12-OH-NVP, 12-hydroxy-nevirapine; NS, not significant; NVP, nevirapine.
[a] *Mann-Whitney U* Test, Median [IQR].
[b] The missing values correspond to patients for whom the metabolite concentration was below the lower limit of quantification for the method.
Differences were considered significant if $p<0.05$.

**Table 8 Sex differences in the proportions of the major nevirapine phase I metabolites**

| | | **Men** | **Women** | ***p* value** |
|---|---|---|---|---|
| | **N** | 33 | 19 | |
| **%** | **2-OH-NVP [a]** | 11.1 [8.2-18.5] | 9.6 [7.8-21.9] | NS |
| | **3-OH-NVP [b]** | 5.5 ± 0.4 | 7.5 ± 0.8 | 0.013 |
| | **12-OH-NVP [a]** | 88.2 [79.8-94.7] | 90.8 [83.2-93.8] | NS |
| **%/kg** | **2-OH-NVP [a]** | 0.17 [0.11-0.26] | 0.15 [0.08-0.42] | NS |
| | **3-OH-NVP [b]** | 0.08 ± 0.01 | 0.12 ± 0.02 | 0.001 |
| | **12-OH-NVP [a]** | 1.24 [1.04-1.29] | 1.35 [1.17-1.76] | 0.037 |

2-OH-NVP, 2-hydroxy-nevirapine; 3-OH-NVP, 3-hydroxy-nevirapine; 12-OH-NVP, 12-hydroxy-nevirapine; NS, not significant; NVP, nevirapine.
[a] *Mann-Whitney U* Test, Median [IQR].
[b] *Student's t-Test*, Mean ± SEM
Differences were considered significant if $p<0.05$.

# 4. Discussion and Conclusions

## 4.1. Development and validation of an analytical method

A reversed phase HPLC-UV method for the simultaneous quantification of NVP and its main phase I metabolites in human plasma was developed and validated. This method allows the accurate and precise quantification of NVP and its metabolites in human plasma, with acceptable sensitivity, specificity and recovery.

Although sample stability upon storage at -80°C and through thawing/freezing cycles was not evaluated in the current work, short-term stability of NVP and NVP metabolites in plasma, after repetitive thawing and freezing, and also in extracted samples at room temperature, are well documented (Rowland et al., 2007, Cammett et al., 2009, Vogel et al., 2010).

Even though the analytical method presented herein might be considered time-consuming comparatively with other methods described in the literature (Rowland et al., 2007, Rezk et al., 2008, Cammett et al., 2009, Kunz et al., 2009, Ren et al., 2010, Vogel et al., 2010), it should be noted that NVP only differs from its phase I metabolites by the presence of a hydroxyl group, and the metabolites are regioisomers, merely differing from each other by the position of this group. Thus, the structural similarity between the analytes hampers their separation on a rapid reversed phase chromatographic method. On the other hand, most of the methods described for the quantification of NVP and metabolites are based on mass spectrometry detection methodologies (Rowland et al., 2007, Rezk et al., 2008, Cammett et al., 2009, Kunz et al., 2009, Ren et al., 2010, Vogel et al., 2010), which implies a significant investment in equipment and also high maintenance costs.

Moreover, the LLOQ obtained with the current method is similar to the LLOQs of previously reported analytical methods for quantification of NVP and NVP metabolites that were based on mass spectrometry detection (Rowland et al., 2007, Rezk et al., 2008, Cammett et al., 2009, Kunz et al., 2009, Ren et al., 2010, Vogel et al., 2010). This LLOQ allows the quantification of metabolites at plasma levels that are expected in patients under NVP-based cART (Rowland et al., 2007, Cammett et al., 2009, Kunz et al., 2009, Vogel et al., 2010).

The absence of an internal standard, and the inability of our method to quantify an additional NVP metabolite, 4-COOH-NVP, could be considered disadvantages of this methodology. We attempted to use pirenzepine as internal standard but had to reject it

from the validation analysis because it showed an unacceptable lack of reproducibility through the analytical runs, unlike NVP and its metabolites which always showed a good reproducibility. In any case, the results obtained in the accuracy, precision and recovery assays were excellent, even without an internal standard. Also, the inability to quantify 4-COOH-NVP in our method should not be regarded as a major limitation since this compound, formed through secondary oxidation of 12-OH-NVP, is a minor metabolite (Riska et al., 1999a, Rowland et al., 2007).

In conclusion, the HPLC method presented herein may be a useful analytical tool for the biomonitoring of NVP and its metabolites and may be applied in studies aimed to explore NVP pharmacology, biotransformation and toxicology.

## 4.2. Exploring sex differences on nevirapine biotransformation in HIV-infected patients

NVP is a remarkable example of a sexually dimorphic profile of adverse drug reactions, with women being at greater risk of experiencing skin and liver toxicity. It has recently been hypothesized that NVP biotransformation plays an important role in the onset of these adverse effects (Pereira et al., 2012a). However, sex-dependent differences in NVP pharmacokinetics have been poorly explored. In the present work, sex differences in the biotransformation profile of NVP were studied in order to explore their potential role in NVP toxicity. We found that the NVP phase I metabolite profile was dissimilar between men and women, with differences being most noteworthy for the 12-OH-NVP and 3-OH-NVP metabolites, which had higher plasma levels in women. Pharmacokinetic variation has been implicated as the main factor underlying the increased rate and wider range of drug-induced toxicity reactions in women (Miller, 2001, Anderson, 2008). These effects have often been purported to be related to higher drug bioavailability in females (Miller, 2001, Umeh and Currier, 2006, Ofotokun et al., 2007, Anderson, 2008). These pharmacokinetic differences may arise due to variations in endogenous and exogenous hormones, and also in liver metabolism (Miller, 2001). Moreover, body size and fat composition are also thought to contribute. Females typically have a lower body weight

and size than males, and also a higher percentage of body fat, which might influence the distribution volume of drugs, in particular those that are highly lipophilic, such as NVP (Anderson, 2008). In fact, a relationship between lower body weight, lower NVP clearance and higher NVP toxicity has been described (de Maat et al., 2002, Kiertiburanakul et al., 2008, Schipani et al., 2011). However, the attempts to demonstrate that patients experiencing higher plasma NVP levels are at greater risk for NVP toxicity have failed, as divergent results have been obtained (de Maat et al., 2003, Almond et al., 2004, Dailly et al., 2004, Kappelhoff et al., 2005, Hall and MacGregor, 2007, Stohr et al., 2008, Wyen et al., 2008, Dong et al., 2012, Ratanasuwan et al., 2012). Nonetheless, it is important to highlight that body weight adjustment was rarely performed in these studies (Almond et al., 2004, Dailly et al., 2004, Kappelhoff et al., 2005, Hall and MacGregor, 2007, Wyen et al., 2008). As expected, in our study population women had lower body weights than men. Additionally, the sex differences found in NVP biotransformation were more pronounced when normalization per unit body weight was performed. This excludes lower body weight as the only factor explaining the different NVP biotransformation between the sexes. The absence of a correlation between NVP concentration and toxicity (Almond et al., 2004, Dailly et al., 2004, Kappelhoff et al., 2005, Hall and MacGregor, 2007) might suggest that NVP *per se* is not toxic but can form toxic metabolites upon biotransformation (Pereira et al., 2012b). NVP is biotransformed into several hydroxylated metabolites (Figure 2) via phase I cytochrome P450 mediation (Figure 3). Females have higher CYP 3A4, 2A6 and 2B6 activities, while sex differences in CYP 2C9 and 2D6 have not been described (Anderson, 2008, Scandlyn et al., 2008, Sinues et al., 2008). Moreover, evidence obtained from pharmacogenetic data has suggested an influence of CYP 2C19 on NVP plasma levels, (Lehr et al., 2011) although without specifying the particular metabolite(s) generated. This CYP isoenzyme has also been linked to the generation of reactive metabolites capable of binding to GSH and forming NVP–GSH adducts in vitro (Wen et al., 2009). Recently, the involvement of CYP 2C19 in the formation of 12-OH-NVP was excluded, (Grilo et al., 2013) but its influence in the generation of the other phase I metabolites was not assessed. The reported higher CYP 3A4, 2A6 and 2B6 activities in females are consistent with the higher proportions of 12-OH-NVP and 3-OH-NVP found for women in the current work (Figure 3 and Tables 7 and 8). Previous work by Hall and MacGregor (2007) did not identify any strong

correlation between plasma levels of NVP or its major phase I metabolites and either hepatotoxicity or skin rash events, or sex differences in metabolite proportions. Nevertheless, no adjustment per unit of body weight was performed in that study. Also, it is important to highlight that the authors performed an extraction of the analytes preceded by a glucuronidase treatment step. This step precluded an estimation of the levels of free phase I metabolites, which prevents direct comparison with our data. The formation of glucuronides (Figure 3) is a major route of elimination of NVP phase I metabolites (Riska et al., 1999a). However, the involvement of phase II metabolic pathways, namely sulphonation, cannot be excluded (Pereira et al., 2012b). For instance, the bioactivation of 12-OH-NVP by SULTs has been increasingly indicated as the plausible mechanism for NVP-associated toxicity (Chen et al., 2008, Antunes et al., 2010a, Antunes et al., 2010b, Srivastava et al., 2010b, Caixas et al., 2012, Meng et al., 2013, Sharma et al., 2013a). 12-OH-NVP is a non-reactive metabolite *per se*; however, it can be bioactivated by SULTs in the liver and skin, yielding the reactive species 12-sulfoxy-NVP (Sharma et al., 2013b). Moreover, using 12-mesyloxy-NVP as a synthetic surrogate for 12-sulfoxy-NVP, we have shown covalent binding of this reactive electrophile *in vitro* to several aminoacids (Antunes et al., 2010a), haemoglobin and human serum albumin (Antunes et al., 2010b), as well as nucleosides and DNA (Antunes et al., 2008). We have also demonstrated for the first time the presence of 12-OH-NVP-derived haemoglobin adducts in HIV-infected patients (Caixas et al., 2012). More recently, Sharma *et al.* (2013b) showed covalent binding of 12-sulfoxy-NVP to skin proteins after incubation of this reactive metabolite with skin homogenate. Likewise, recent work by Meng *et al.* (2013) showed evidence for the formation of NVP–human serum albumin adducts, consistent with reaction with 12-sulfoxy-NVP. Nevertheless, the detection of 12-sulfoxy-NVP in man has not yet been achieved. Recent evidence has shown that recombinant human SULT 1A1*1 is capable of converting 12-OH-NVP into 12-sulfoxy-NVP (Sharma et al., 2013b). Owing to its broad spectrum of substrates and high hepatic and extra hepatic expression, SULT 1A1 appears to be the main form of human SULT involved in the detoxification of xenobiotics, particularly phenolic metabolites (Glatt, 2000). Nonetheless, further studies are needed to clarify whether other SULTs play a role in the bioactivation of 12-OH-NVP (Michaud et al., 2012) (Figure 3), as well as whether the isoforms involved are the same in skin and liver. It is known that

SULT 1A1 is highly polymorphic and that there are marked differences in the activities of SULT 1A1 variants (1A1*1, 1A1*2 and 1A1*3) (Nagar et al., 2006). These differences may determine distinct susceptibilities to NVP toxicity and also the tissue-specific responses. While it is difficult to explain sex-related differences on the basis of genetic polymorphisms, as there is no evidence for a sex-dependent pattern in the frequencies of polymorphic SULTs, sex-dependent enzymatic regulation of SULTs or differences in PAPS availability are plausible. Little is known about the sexually dimorphic expression of SULTs in humans, but sex-divergent SULTs are mostly female predominant in mice; (Alnouti and Klaassen, 2011) for instance, female mice showed higher hepatic mRNA levels of SULT 1A1 compared with male mice (Alnouti and Klaassen, 2006, Alnouti and Klaassen, 2011, Suzuki et al., 2012). Likewise, higher expression of SULT 1D1 was reported in the canine female liver (Tsoi et al., 2001). In addition, a female predominance in SULT 2A1/2A2 has been reported in mice (Wu et al., 2001, Alnouti and Klaassen, 2006) and rats (Liu and Klaassen, 1996, Dunn and Klaassen, 1998). It is also noteworthy that Alnouti and Klaassen (2011) demonstrated that androgens and a male pattern of growth hormone secretion can have a suppressive effect on the expression of some SULTs in mouse hepatic tissue, while oestrogens and a female pattern of growth hormone secretion can exert opposite effects. Furthermore, the activity of human SULT 2 enzymes, including SULT 2B1b, which is expressed in the skin and is capable of sulphonating a number of xenobiotics, has been shown to undergo modulation by several types of nuclear receptors; among these are peroxisome proliferator-activated receptors (PPARs) (Runge-Morris et al., 2013). It has been argued that metabolic interactions between PPARɣ or PPARα and oestrogens, oestrogen receptors or oestrogen receptor-related cofactors could explain, at least in part, some sex-specific differences observed in PPAR-based treatments (Benz et al., 2012). Whether or not a sexually dimorphic pattern in PPAR expression is an underlying cause of differential NVP toxicity remains to be established. In addition to 12-OH-NVP, current evidence suggests that 3-OH-NVP might also undergo bioactivation (Srivastava et al., 2010b); however, there is no clear evidence so far associating 3-OH-NVP or any 3-OH-NVP derivative with NVP-related toxic reactions. Interestingly, in the present study, sex differences were found only for the plasma levels of 3-OH-NVP and 12-OH-NVP, which is consistent with the hypothesis of sex-dependent formation of reactive metabolites. Also, the fact that women have lower UDP-glucuronosyltransferase

activity (Anderson, 2008, Gallagher et al., 2010) suggests that they may be prone to less efficient detoxification of these metabolites than men. Sex-dependent variations in the expression/activity of PAPS synthase enzymes, which catalyse the biosynthesis of the SULT cofactor PAPS, may also contribute to the different toxicity outcomes. PAPS is the universal donor of the sulphonyl moiety that enables the sulpho-conjugation of SULT substrates. At least three PAPS synthase isoforms (PAPSS1, PAPSS2a and PAPSS2b), with different activities and tissue distributions, have been identified in humans (Fuda et al., 2002). While PAPSS2b is the main isoform in human liver, PAPSS2a is not expressed in this tissue and PAPSS1 is expressed to a lesser extent in the liver compared with several other tissues (Fuda et al., 2002). A similar tissue distribution of PAPS synthase isoforms has been reported in mice and, interestingly, the hepatic expression of PAPSS2 in mice has been found to be female predominant (Alnouti and Klaassen, 2006). Although sex-related differences in the expression of PAPS synthase enzymes do not appear to have been investigated in humans, it is noteworthy that both PAPSS1 and PAPSS2 are highly polymorphic (Xu et al., 2002, Xu et al., 2003) and are differentially expressed in various tissues (Venkatachalam, 2003). In particular, PAPSS1 expression is notably high in the skin (Venkatachalam, 2003), where sulphonation of 12-OH-NVP has been associated with NVP-induced skin rash (Sharma et al., 2013b). It should also be noted that the role of immune-mediated responses in the onset of NVP-related toxic reactions is well recognized, with higher $CD4^+$ cell counts being associated with a higher risk of hepatotoxicity and skin rash (Martin et al., 2005 , Medrano et al., 2008). The predominance of autoimmune diseases in women, who have stronger immune responses than men, is widely documented (Voskuhl, 2011, Tiniakou, 2013). While this is likely to contribute to the sex-dependent profile of adverse events related to NVP treatment, current therapeutic decisions already take some of these effects into account. In fact, the introduction of lower $CD4^+$ cut-off level criteria for the initiation of NVP therapy in women than in men has led to a decreased incidence of drug-related toxicity (Thompson et al., 2010). Nonetheless, reports of adverse effects still persist, suggesting that other factors are at play. Thus, in addition to distinct immune responses, sex-related differences in metabolic activation may contribute to the sexually dimorphic profile of adverse events related to NVP treatment. NVP is among an increasing number of drugs found to display sex differences in pharmacokinetics and in adverse events upon biotransformation.

Despite significant progress in recent years, there are still large gaps in our knowledge of the effects of sex upon the clinical pharmacokinetic of NVP. Although our present work provides evidence for a sex-dependent dimorphism in NVP metabolism, it does not appear that differences in the concentrations of NVP and its phase I metabolites *per se* are the basis for the apparently higher risk of idiosyncratic reactions to NVP in women. Future research must be conducted to elucidate the role of SULTs in NVP metabolism and bioactivation, and on the elicited sex-related differences in susceptibility to the toxicity of the drug, with a view to the personalized, safer prescription of NVP.

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